Characterization of Phospholipids in Membrane Vesicles Derived from Pseudomonas aeruginosa

Yosuke Tashiro,* Aya Inagaki, Motoyuki Shimizu, Sosaku Ichikawa, Naoki Takaya, Toshiaki Nakajima-Kambe, Hiroo Uchiyama, and Nobuhiko Nomura†

Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

Received October 21, 2010; Accepted December 7, 2010; Online Publication, March 7, 2011 [doi:10.1271/bbb.100754]

Many Gram-negative bacteria release membrane vesicles (MVs), but their phospholipid properties are poorly understood. Phosphatidylglycerol was present at high levels in MVs derived from Pseudomonas aeruginosa, but not in the cellular outer membrane. The ratio of stearic acid in MVs was high compared to that in the cellular outer membrane. These findings suggest that membrane rigidity is associated with MV biogenesis.

Key words: Pseudomonas aeruginosa; membrane vesicles; phospholipid; fatty acid

The bacterial membrane plays key roles both as a barrier between living cells and their environment and in helping bacteria adapt to new conditions to promote survival. Changes in the composition of acyl chains or head groups can alter bilayer fluidity and stability, and this alteration is important in response to environmental perturbations. The membrane lipid bilayer undergoes a reversible change in state during a disordered and an ordered array of fatty acid chains, and changes in fluidity. Recent studies have confirmed the heterogeneity of phospholipids in bacterial membranes.

Many Gram-negative bacteria produce membrane vesicles (MVs), which are secreted from outer membranes (OMs) into the external milieu. MVs are bilayered spheres 20–200 nm in diameter and consist of phospholipids, lipopolysaccharides (LPSs), OM proteins (OMP), and periplasmic constituents. They have been studied in many pathogens because they contain a wide variety of virulence factors. Pseudomonas aeruginosa, a common environmental bacterium and an opportunistic human pathogen, is one of the most well-studied bacteria in terms of MV characterization. P. aeruginosa MVs are also carriers of quorum-sensing signal molecules, components of biofilm matrices, and secretion systems discarding misfolded OM proteins from the periplasm.

Previously, studies in this area have extended the understanding of MV pathogenicity, its biological functions, and its production mechanisms in many bacteria, including P. aeruginosa. However, the mechanisms of MV formation and their physiological roles have not been clearly identified. While biochemical and biological approaches have been utilized in MV studies, little attention has been given to the physiochemical properties of MVs. To understand better the roles of MVs, the physiochemical properties of phospholipids contained in MVs must be clarified comprehensively. In this study, we analyzed the phospholipid composition of MVs derived from P. aeruginosa to gain insight into the biogenesis and physiological roles of MVs.

P. aeruginosa PAO1 was cultured in 500-mL flasks containing 100 mL of Luria-Bertani (LB Lennox) medium with shaking at 150 rpm at 37 °C. At the early stationary phase (culture time 5 h), the cells and supernatants were separated, and cellular OM and MVs were purified. Cellular OM was purified as described previously. By this method, the OM was purified as inside-out OM vesicles. MVs were purified from the supernatant by a previously described method. Hydrodynamic diameters were measured using the light scattering method by Zetasizer Nano ZS (Malvern Instruments, Malvern, UK), as described previously, and the average diameter was 126.9 ± 0.7 nm (n = 3) for purified cellular OM and 111.6 ± 0.9 nm (n = 3) for purified MVs, suggesting that both formed spheroid substances.

To determine the composition of head groups of glycerophospholipids, lipids were extracted from cellular OM and MVs as previously described and were analyzed by thin layer chromatography (TLC). To avoid the effects of several metals in the TLC assay, EDTA-2Na (pH 8.0) was added to each sample at a final concentration of 1 mM before lipid extraction. Both cellular OM and MVs contained at least three main glycerophospholipids, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylcholine (PC). The most abundant glycerophospholipid in the cellular OM of P. aeruginosa was PE (Table 1), suggesting that the glycerophospholipid composition of PE is similar to previously reported data on P. aeruginosa phospholipid composition. However, MVs derived from P. aeruginosa contained more PG than PE (Table 1), suggesting that the glycerophospholipid com-

---

* Present address: Division of Environmental Engineering, Faculty of Engineering, Hokkaido University, Kita 13, Nishi 8, Kitaku, Sapporo, Hokkaido 060-8628, Japan
† Abbreviations: LPS, lipopolysaccharide; MV, membrane vesicle; OM, outer membrane; OMP, outer membrane protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PQS, Pseudomonas quinolone signal; SFA, saturated fatty acid; TLC, thin layer chromatography; UFA, unsaturated fatty acid

---
Table 1: Phospholipid Composition in the Cellular OM and MVs*  

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Cellular OM</th>
<th>MVs</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>59.7 ± 2.9</td>
<td>33.1 ± 0.8</td>
</tr>
<tr>
<td>PG</td>
<td>27.1 ± 1.7</td>
<td>63.0 ± 1.2</td>
</tr>
<tr>
<td>PC</td>
<td>13.2 ± 2.8</td>
<td>3.9 ± 1.1</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*Normalized percentages of total PE, PG, and PC are shown. Extracted crude phospholipids at the early stationary phase were spotted onto an aluminum-backed Silica Gel 60 TLC plate (Merck, Darmstadt, Germany) and separated in a 65:25:4 mixture (by volume) of chloroform:methanol:water. TLC spots were identified according to the migration of phospholipid standards. The intensities of the spots were measured with a UV lamp after spaying of a primulin solution (0.01 mg in 100 mL of acetone:water, 3:2), and were evaluated by densitometric scanning using Image Master ID Elite (GE Healthcare, Buckinghamshire, UK). Data shown are means ± standard deviations of three independent determinations.

Table 2: Fatty Acid Composition of Phospholipids in the Cellular OM and MVs*  

<table>
<thead>
<tr>
<th>Growth stage</th>
<th>Early stationary phase</th>
<th>Late stationary phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane</td>
<td>Cellular OM MVs</td>
<td>Cellular OM MVs</td>
</tr>
<tr>
<td>C16:0</td>
<td>43.4 ± 1.8</td>
<td>33.5 ± 2.9</td>
</tr>
<tr>
<td>C16:1</td>
<td>13.5 ± 2.5</td>
<td>5.0 ± 2.0</td>
</tr>
<tr>
<td>C17:1</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td>C18:0</td>
<td>6.5 ± 0.6</td>
<td>37.9 ± 4.8</td>
</tr>
<tr>
<td>C18:1</td>
<td>36.4 ± 2.8</td>
<td>22.7 ± 3.4</td>
</tr>
<tr>
<td>C19:1</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td>SFA</td>
<td>49.9</td>
<td>71.4</td>
</tr>
<tr>
<td>UFA</td>
<td>49.9</td>
<td>28.6</td>
</tr>
</tbody>
</table>

*Normalized percentages of total fatty acids are shown. C16:0, palmitic acid; C16:1, palmitoleic acid; C17:1, cis-10-heptadecenoic acid; C18:0, stearic acid; C18:1, oleic acid; C19:1, cis-10-nonadecenoic acid. The fatty acid methyl esters (lower layer) were fractionated using a Shimadzu GC-2010 (Shimadzu, Kyoto, Japan) gas chromatograph equipped with a mass spectrometer GCMS-QP 2010 (Shimadzu). The GC operating conditions were as follows: the GC column DB-5 (Agilent J&W, Folsom, CA) was held at 60°C for 1 min, heated at 2°C per min to 300°C, and then held for 6 min at that temperature. Fatty acid methyl esters were identified by comparing their retention times with standards. Data shown are means ± standard deviations of three independent determinations. BDL, below detection limit.

Components of MVs are different from those of the cellular OM. The phospholipid composition of E. coli MVs was previously reported by Horstman et al. to be similar to that of the E. coli OM.14) The different results as between E. coli and P. aeruginosa indicate that MV formation is not uniform across bacterial species. It is known that PE more greatly affects lipid phase transition compared to C16 fatty acids, while they were high in MVs at both growth phases (Table 2). Longer acyl-chains of fatty acids are known to decrease membrane fluidity.13,14) Hence, it is suggested that MVs have rigid membrane properties.

A relationship between membrane rigidity and MV formation has been proposed by Mashburn-Warren et al.17) In P. aeruginosa, a quorum-sensing molecule, 2-heptyl-3-hydroxy-4-quinolone (Pseudomonas quinolone signal [PQS]), not only is present in MVs, but also enhances MV production. Since PQS interacts with LPS and causes LPS to remain more ordered, it has been hypothesized that high fluidity in membranes prevents the development of the curvature necessary for MVs. Our results indicate that the percentages of SFAs and long-chain fatty acids are high in MVs (Table 2), and that this is related to a decrease in membrane fluidity. This confirms the hypothesis that MVs are formed at a site where the membrane is relatively rigid. In the recent study, we found that the amount of PQS in MVs at the late stationary phase is higher than at the exponential or the early stationary phase and suggests that the mechanism of MV production is not uniform across the several growth phases.5) However, membrane rigidity in MVs was observed at both the early and the late stationary phase (Table 2). Hence, membrane rigidity is considered to be associated with MV biogenesis irrespective of the growth phase transition. Since the rigid membrane does not appear to adapt to changes in environment, it is of interest if the release of MVs is a strategy to increase the fluidity of the OM, but the detailed mechanism remains unknown, and further analysis is needed to understand the mechanism of MV biogenesis.

In conclusion, this report is the first to describe the phospholipid properties of MVs in P. aeruginosa. We found that P. aeruginosa MVs have unique characteristics in terms of phospholipid composition and fatty acid constituents. MVs possessed high ratios of PG and stearic acids, and their compositions were quite different from P. aeruginosa cellular OM. These results suggest that membrane rigidity is associated with membrane biogenesis.

Acknowledgment

This study was supported in part by a Grant-in-Aid (21380056) for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to N. N.).

References


in its membrane lipids, the membrane lipids of MVs contained only 28–29% UFAs at both growth phases (Table 2). It is known that phospholipids that contain UFAs have much lower transition temperatures than those composed of saturated fatty acids (SFAs).1 Another notable point is that the percentages of C18 fatty acids were relatively low in cellular OM as compared to C16 fatty acids, while they were high in MVs at both growth phases (Table 2). Longer acyl-chains of fatty acid are known to decrease membrane fluidity.13,14) Hence, it is suggested that MVs have rigid membrane properties.

A relationship between membrane rigidity and MV formation has been proposed by Mashburn-Warren et al.17) In P. aeruginosa, a quorum-sensing molecule, 2-heptyl-3-hydroxy-4-quinolone (Pseudomonas quinolone signal [PQS]), not only is present in MVs, but also enhances MV production. Since PQS interacts with LPS and causes LPS to remain more ordered, it has been hypothesized that high fluidity in membranes prevents the development of the curvature necessary for MVs. Our results indicate that the percentages of SFAs and long-chain fatty acids are high in MVs (Table 2), and that this is related to a decrease in membrane fluidity. This confirms the hypothesis that MVs are formed at a site where the membrane is relatively rigid. In the recent study, we found that the amount of PQS in MVs at the late stationary phase is higher than at the exponential or the early stationary phase and suggests that the mechanism of MV production is not uniform across the several growth phases.5) However, membrane rigidity in MVs was observed at both the early and the late stationary phase (Table 2). Hence, membrane rigidity is considered to be associated with MV biogenesis irrespective of the growth phase transition. Since the rigid membrane does not appear to adapt to changes in environment, it is of interest if the release of MVs is a strategy to increase the fluidity of the OM, but the detailed mechanism remains unknown, and further analysis is needed to understand the mechanism of MV biogenesis.

In conclusion, this report is the first to describe the phospholipid properties of MVs in P. aeruginosa. We found that P. aeruginosa MVs have unique characteristics in terms of phospholipid composition and fatty acid constituents. MVs possessed high ratios of PG and stearic acids, and their compositions were quite different from P. aeruginosa cellular OM. These results suggest that membrane rigidity is associated with membrane biogenesis.

Acknowledgment

This study was supported in part by a Grant-in-Aid (21380056) for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to N. N.).

References